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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Arnvidarson	Examiner: Zeman, Mary K.
Serial No.	: 09/830,558	Art Unit: 1631
Filed	: September 24, 2004	Attorney's docket: P66611US0

Title : A system for regulating the handling of milk during the milking process and a method for regulating said milking process

DECLARATION BY BÖRKUR ARNVIDARSON UNDER 37 C.F.R. § 1.132

I, Börkur Arnvidarson, declare and state as follows:

1. I am employed as project manager at Chemometec A/S, Allerød, Denmark

2. I am educated as a chemist and chemical engineer and have been working in the field of analytical chemistry since 1988. Through the years I have had employment in the field of analytical chemistry, including a four years employment at Foss Electric, which is the world leading producer of analytical instruments to the dairy industry.

3. I am inventor of the above-identified U.S. application. I have reviewed the Office Action of June 29, 2004.

4. To my knowledge, only two *automated instruments* for the counting of somatic cells in milk samples were available in 1993, namely the Fluoro-opto-electronic method (Fossomatic, Foss Electric, DK), described as Method C in the International IDF standard 148A:1995 and, at the time, a newly adapted Fluoro-opto-electronic method of flow cytometry (SomaCount, Bently Instruments, USA).

5. The analysis of the number of somatic cells using the Fluoro-opto-electronic methods comprises in practice the following steps:

   - a cow is milked using a milking installation,
   - a representative milk sample is collected during the milking, either manually or automatically,
   - the sample is transferred into a sample container, where preservative is added and the sample is cooled,
   - the sample is transported to a regional central analysis laboratory,
   - the sample is heated to the melting point of milk fat and mixed thoroughly,
   - the sample is mixed with a volume of reagent,
   - a cell count of the sample is provided by an instrument, such as the Fossomatic instrument, which is based either on the principle of dispersing a thin film (appr. 0.05 x 3 mm) onto the edge of a highly polished rotating disk (where a fixed strip of the film is analysed), or by generating a thin stream of liquid (less than 0.01 mm) arranged in the centre of a carrier stream (flow cytometry),
   - the individual cells are counted as a short variation in fluorescence intensity (peak of appr. 1 □s) when the stream of milk and reagent passes by a detector (a photon sensitive photo multiplier) and is exited with an intense light source (generally a high power lamp or a laser),
   - the cell count of the analysed milk and reagent mixture is determined by dividing the number of identified fluorescence peaks by an estimate of the analysed volume,

- the result of the determination is communicated back to the farm.

6. In contrast, the method according to the present patent application comprises the following steps:

   - a cow is milked using a milking installation,
   - during the milking process, the milk stream, or a part thereof, is passed through a sample domain, optionally mixed with a dye,
   - an on-line, real-time assessment of particles is performed,
   - a comparison of the result of the particle assessment with a predetermined quality parameter is made, and
   - the result of the comparison determines to which outlet the milking stream is to be directed.

   The step of initiation of the milking is described in Example 1 of the application. The direction of the milking stream, or part thereof, through the particle assessment apparatus is described on page 15, lines 6-16.

   The step of particle assessment is described in Example 2. A preferred way to carry out this assessment is the following:

   > A representative volume of the milk being milked is placed in a sample domain (measuring chamber) where detection of specific signals is possible, e.g. between two sheets of glass, held apart by a spacer forming a suitable gap of for instance about 0.1 mm.
   >
   > The sample is then illuminated with light suitable for the excitation of molecules, e.g. a DNA staining dye added to the sample, preferably by low power light source, such as Light Emitting Diodes, and an image of the sample compartment, or fixed part of the sample compartment, is recorded using a Charge Coupled Device camera (detection time of about 1 second).
   >
   > The volume thus analysed in a single image is sufficiently large to allow the determination of the number of specific particles with sufficient precision to allow successful regulating of the milking process, preferably by using optical arrangement with low or no linear enlargement, or even reduction, thus effectively allowing the detection of signals in large sample compartments (e.g. 5x6x0.1mm) equivalent to the investigation of large sample volumes (typically more than 1 □).

   The physical implementation of such a detector results in an instrument small in size, comparable to a shoebox, e.g. with dimensions of about 20x15x25 cm.

   The subsequent step of comparison of the result of the particle assessment with a predetermined quality parameter is described on page 12, lines 6-13 of the application. The regulation of the milking stream on the basis of the comparison is described on page 14, lines 20-34. The analysed milk can go back to the milking stream or be discarded.

7. Thus, the method of the patent application provides an on-line analysis of the particles in the milk, allowing immediate discrimination between bad quality milk and good quality milk. Any transportation to and analysis by specialized sophisticated laboratory equipment is no longer necessary.

8. The Fluoro-opto-electronic methods known at the time the patent application was filed, could not be incorporated into an on-line milking process for the following reasons:

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- the fluoro-opto-electronic method requires a very sensitive apparatus, not suitable for use at a milking farm The apparatus has the following fragile features: Flow system to generate a stable and known stream of a milk and reagent mixture. The detection of fluorescence from a particle is done in a short period of time, demanding sensitive detection means (e.g. photo multipliers) as well as powerful source of excitation light (e.g. high power lamp or laser).

- if to be used in a milking system on a farm, the Fossomatic apparatus or flow cytometer would need to be placed in the same environment as the animals. Placement in a separate room would, apart from imposing further costs, not be feasible as samples need to be transported to the apparatus. In a fully automatic system, this would require tubes. Such tubes would need to be of considerable length and thus contain a large 'dead' volume relative to the total volume of milk obtained in one milking. This would not be a workable solution. Placing such an apparatus in a stable is not a workable solution either. A milking stable environment is characterized by changing temperatures, changing humidity, presence of corrosive vapours, insects and mammals (e.g. mice or rats) and considerable risk for physical damage, etc. This is not a suitable setting for placing such equipment.

- apparatuses used for the fluoro-opto-electronic method require regular calibration and monitoring. Several of the critical components, such as the generation of the flow of particles, detection and excitation of fluorescent light have direct influence on either the intensity of detected signals or size of analysed volume. Instrumental drift is therefore a known problem of these instruments, compensated for by frequent measurement of calibration samples (every 10 to 20 minutes) compared to the frequency of milking (one sample every 2 to 10 minutes). Use in a farm setting requires a robust system, not suffering from drift problems.

- Fossomatic-type or flow-cytometry equipment is too costly for application in a method or system for regulating a milking process. The price of an apparatus for routine analysis that is currently used in centralized laboratories that analyze milk samples is in the order of USD 100,000 – 200,000, i.e. more than the costs of an entire milking installation. Even if a less accurate version would be used, its costs would amount to at least ca. USD 35,000. To this costs for calibration and maintenance to be carried out by skilled persons would have to be added.

- apparatuses used for the fluoro-opto-electronic method may be clogged by large particles. This may be acceptable for the off-line analysis of samples, where analysis may be interrupted for cleansing the system and/or re-testing, but it would obviously be destructive in a real-time system for regulating a milking process.

- Fossomatic-type or flow-cytometry instruments are generally large in size (e.g. 150x70x50 cm) and thus difficult to place in close proximity of the milking station.

As a conclusion it is evident that at the time of patenting, the application of the state of the art automated fluoro-opto-electronic methods for the purpose of generating automated real-time cell counting results, with the purpose of regulating milking is impossible, and further no records of such attempts are known by the undersigned, despite the fact that these methods have existed in the past.

Date: Oktober 22 2004 Signature: Börkur Arnvidarson

(Börkur Arnvidarson)

Börkur Arnvidarson - Curriculum Vita

1981-1984	University of Iceland, B.Sc. in Chemistry 1984.
1984-1986	Researcher at The University of Copenhagen, inst. Physical Oceanography. The work " Fluorescence in Relation to Oceanographic Problems" awarded "Gold Medal" from the University of Copenhagen.
1986-1988	Danish Technical University (DTU). M.Sc. Chemical Engineering. The final project in Flow Injection Analysis, from the University of Washington, Seattle, Prof. Jaromir Ruzicka.
1988	Participation in graduate course, Chemometrics. Bruce Kowalski, University of Washington, Seattle.
1988-1990	Employed at the "Nordic Institute for Paint and Polymer Research". Chemical analysis of paint and polymers, based on chromatography (HPLC, GPC, GC) and spectroscopy (FT-IR, UV/Vis, AA, EDS)
1989-1990	Daily manger of the analytical laboratory. Responsible for all chemical analysis, including criminal analysis of paint and polymers for the Danish Criminal Police.
1989-1990	Lecturer in the course "Paint as Technical Evidence", given for the Danish Criminal Police.
1990-1991	Employed as an independent consultant, in the fields of chemometrics, statistics, data collection and software programming. Involved in work for R&D departments of a number of large companies in Denmark and Scandinavian.
1991-1995	Employed at Foss Electric A/S in R&D department. Working with analytical chemistry and spectroscopy. Responsible for the implementation of chemometrics in a composition analyser for milk.
1994-1995	Responsible for the implementation of chemometrics in Foss Electric A/S
1993-1994	Specialist for a working group under the EU-agricultural council. Advisor in the statistical interpretation of the results from routine analysis.
1995-1996	Employed as an independent consultant, in the fields of chemometrics, statistics, data collection and software programming.
1996	Co-founder and CEO of ChemoMetec A/S.

1998 Project Manager at ChemoMetec A/S responsible for particle detection and statistical methods.

English Version

INTERNATIONAL DAIRY FEDERATION

INTERNATIONAL IDF STANDARD 148A:1995

IDF: 41, SQUARE VERGOTE, B - 1040 BRUSSELS (BELGIUM) Price: 500 Belgian Francs

MILK
ENUMERATION OF SOMATIC CELLS

METHOD A – MICROSCOPE METHOD (Reference method)

1 SCOPE

This International Standard specifies the reference method for counting somatic cells in both raw and chemically preserved milk.

It is suitable for preparing standard test samples and for calibrating mechanized and automatic cell-counting procedures.

2 REFERENCE

IDF 50B: 1985 - Milk and milk products - Methods of sampling.

3 DEFINITION

For the purpose of this International Standard, the following definition applies.

Somatic cells: Those cells with nuclei, for example, all leucocytes and epithelial cells.

4 PRINCIPLE

Spreading of the milk (0,01 ml) to be examined over a slide (1 cm²). Drying and staining of the film and subsequent counting of the stained cells using a microscope. Multiplication of the number of cells counted in a defined area by a working factor to give the number of cells per millilitre.

5 REAGENTS

All reagents shall be of recognized analytical quality. Water used shall be distilled or deionized water, or water of at least equivalent purity.

5.1 Dye solution

Warning.- Tetrachlorethane is poisonous.

Preparation and application of the dye solution shall be carried out in a fume cupboard.

Composition

Ethanol, 95% (V/V)	54,0 ml
Tetrachlorethane	40,0 ml
Methylene blue	0,6 g
Acetic acid, glacial	6,0 ml

Note.- As an alternative, tetrachlorethane may be replaced by the same amount of trichlorethane. Instead of methylene blue ethidium bromide can be used (see Method C).

Preparation

Mix the ethanol and tetrachlorethane in a bottle and heat in a water bath (6.1) to 60-70°C. Add the methylene blue, mix carefully, cool in a refrigerator to 4°C and then add the glacial acetic acid. Pass the solution through an appropriate filter (6.3) and store it in an airtight bottle. If necessary, filter again before use.

First, second and third editions

Following a resolution at the IDF Seminar on Mastitis Control at Reading, England, in April 1975, the IDF Group of Experts on Mastitis (Group A2) decided to set up a subgroup to consider aspects of somatic cell counting in relation to bovine mastitis. The subgroup's draft recommended procedure for cell counting was first published in the Bulletin of IDF Document 114/1979.

However somatic cell counting procedures were kept under review by Group A2 and the Cell Count Subgroup A2B was re-activated to revise the first edition. Additional information on the Fossomatic and Auto-Analyzer methods of somatic cell counting in milk was included and the second edition published in the Bulletin of IDF Document 168/1984.

A third edition was prepared in the light of further experience and issued as IDF Standard 148:1991.

Fourth edition

This fourth edition has been prepared by the somatic cell count subgroup (A2B/E59) comprising the following members:
J.M. Booth (GB), Chairman, W. Heeschen (DE), A. Saran (IL), W.M. Schällibaum (CH), L.O. Sjaunja (SE), H. Van Hemert (NL), L.S. Hinckley (US), Ch. Chan (AU), W.H. Giesecke (ZA), R. Franks (NZ), P. Schmidt Madsen (DK).

It was approved for publication (Report A-Doc 170) at the IDF Annual Sessions in Adelaide, Australia, September 1994 as a final standard. This standard is not produced jointly with ISO and AOAC.

This standard supersedes IDF Standard 148:1991.

IDF General Secretariat
May 1995

6 APPARATUS

Usual laboratory equipment, and in particular:

6.1 Water bath, capable of being maintained at 60-70°C.

6.2 Water bath, capable of being maintained at 30-40°C.

6.3 Filter with a pore size of 10-12 µm or less.

6.4 Microscope, magnification 500-1000x.

6.5 Microsyringe, of capacity 0,01 ml, with maximum tolerance of 2%.

6.6 Slides, with the shape of a film marked of 20 mm x 5 mm, or a standard slide and template with the shape of a film of 20 mm x 5 mm.

6.7 Hotplate, capable of being maintained at 30-50°C.

6.8 Fan, hairdryer type.

Note.- If ethidium bromide is used the microscope must have a fluorescence equipment.

7 SAMPLING

7.1 See IDF Standard 50B: 1985.

7.2 When automatic samplers are used, they shall have been properly tested.

7.3 If samples are to be stored prior to testing or preservation, this shall be done at storage temperatures of 2-6°C.

7.4 If samples are not to be tested within 6 h of sampling, they shall be preserved by addition of boric acid. The final concentration of boric acid in the sample shall not exceed 0,6 g/100 ml; such samples may be stored for no longer than 24 h at 2-6°C.

8 PREPARATION OF TEST SAMPLE

Heat the laboratory sample in a water bath (6.2) to 30-40°C. Then mix carefully and cool to the temperature at which the microsyringe has been calibrated, for example, 20°C.

9 PROCEDURE

9.1 From each test sample at least two films shall be prepared and counted.

Clean the slides (6.6), for example with ethanol, dry with dust-free paper, flame and cool.

9.2 Test portion

Take 0,01 ml of the prepared test sample (8) using a microsyringe (6.5). Carefully clean the outside of the syringe in contact with the milk.

9.3 Place the test portion on the clean slide, first drawing the outline of the shape (20 mm x 5 mm). Then fill in the area as evenly as possible. Dry the film on a levelled hotplate (6.7) until completely dry. Better results can be obtained by drying the films at ambient temperature for several hours.

9.4 Dip the dried film on the slide in the dye solution (5.1) for 30 min. Complete drying with the fan (6.8) if required. Then dip the film in tap water until all surplus dye is washed away. Dry again and store with protection against dust.

9.5 Determination

Using the microscope (6.4), count the cell nuclei in the film (at least 400). These are clearly recognizable and at least half should be visible in the microscopic field. Avoid counting strips selected exclusively from the peripheral areas of the film.

Check at least once a month the proper preparation of the films, and hence the reliability of the results, by counting different parts of the film.

10 EXPRESSION OF RESULTS

10.1 Method of calculation

10.1.1 The number of somatic cells counted is multiplied by the «working factor» to give the number of cells per millilitre of milk.

10.1.2 Calculation of the working factor

The length of the strips to be counted is 5 mm each. The breadth of a strip corresponds to the diameter of the microscope field. Using 0,01 ml of milk, the working factor then is

$$\frac{20 \times 100}{d \times b}$$

where

d is the diameter, in millimetres, of the microscope field;

b is the number of strips counted completely

11 PRECISION

11.1 See Appendix 1.

11.2 Minimum number of cells to be counted

Since microscopic counting of somatic cells may also be used for calibration of automatic and mechanized counting procedures, the coefficient of variation of counts on identical samples shall not be higher than that of electronic instruments. The coefficient of variation on a milk sample containing 400 000-600 000 cells/ml, with approximately 60% neutrophils, shall not exceed 5%. To meet this requirement, the number of somatic cells to be counted in each sample shall be at least 400.

the Poisson distribution presupposes that

$$M = V = s^2$$

where

M is the mean value;

V is the variance;

s is the standard deviation.

The coefficient of variation is

$$CV = \frac{s \times 100}{M}\% \text{ or } CV = \frac{100}{s}\% \text{ or } CV = \frac{100}{\sqrt{M}}\%.$$

where

M (mean) is the number of particles (cells) that have been counted.

12 TEST REPORT

The test report shall show the method used, the working factor of microscopy, and the result obtained. It shall also mention any operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the results.

The report shall include all details required for the complete identification of the sample.

METHOD B – COULTER COUNTER METHOD

1 SCOPE

This International Standard specifies a method for counting the number of somatic cells in both raw and chemically preserved milk, using the Coulter counter.

2 REFERENCE

IDF 50B: 1985 - Milk and milk products - Methods of sampling.

3 DEFINITION

For the purpose of this International Standard, the following definition applies.

Somatic cells: Those particles that are counted by an electronic particle counter, after fixing a lower threshold level and elimination of fat particles overlapping the size range of somatic cells.

4 PRINCIPLE

Addition of formaldehyde solution (formalin) to the milk to be examined to fix the somatic cells. Dilution by an emulsifying electrolyte mixture and subsequent sufficient heating to break down the fat globules overlapping the size range of the cells.

In an electronic particle counter, the milk passes through an aperture located between electrodes. When a particle passes through this aperture it displaces its own volume of highly conductive liquid by one of lower conductivity. The increased resistance raises the voltage, producing a voltage pulse proportional to the volume of the particle. The number of pulses indicates the number of passing particles. Only pulses above a fixed threshold level are counted.

Direct reading of the number of somatic cells in thousands per millilitre.

Note.- The user of this method should be aware that owing to the counting principle (particle counting) the results are not always comparable with those of Methods A and C.

5 REAGENTS

All reagents shall be of recognized analytical quality. Water used shall be distilled or deionized water or water of equivalent purity.

5.1 Emulsifier electrolyte mixture

Composition	
Ethanol, 96% (V/V)	125,0 ml
Polyethylene glyco mono [p- (1, 1, 3, 3-tetramethyl-butyl)-phenyl]-ether (for example Triton X-100, conc.)	20,0 ml
Sodium chloride solution, 0,9 g/100 ml	855,0 ml

Preparation

Carefully mix the polyethylene glycol mono [p- (1, 1, 3, 3-tetramethylbutyl)-phenyl]-ether and the ethanol. Add the sodium chloride solution. Pass the mixture through an appropriate filter (6.6).

Notes.-

1 *To prevent bacterial growth, 10 ml of formaldehyde solution 35% (V/V) may be added to the emulsifier electrolyte mixture.*

2 *Commercially available emulsifying electrolyte mixture may be used, for example Somaton diluent.*

3 *Daily tests shall be made in order to determine the number of extraneous particles in the emulsifier electrolyte mixture. This mixture and the plastic and glassware are considered sufficiently clean if the number of particles is below 20 per 0,1 ml of emulsifier electrolyte mixture.*

5.2 Fixative liquid

Mix 0,02 g of eosine and 9,4 ml of formaldehyde solution 35% (V/V) and make up with water to 100 ml. Filter or centrifuge the liquid in order to remove particles.

Notes.-

1 *The formaldehyde concentration of commercially available formalin varies between 35 and 40% (V/V). This shall be taken into account when preparing the fixative liquid.*

2 *Eosine is included in the fixative liquid for colouring the fixed test samples.*

6 APPARATUS

Prior to use all glassware shall be carefully cleaned to be as near as possible free from particles.

6.1 Coulter counter

Electronic particle counter with a capillary tube of 100 µm diameter and counting volumes of 0,1 or 0,5 ml (for example Coulter Counter models F or FN*). Alternatively an automatic counter (for example Milk Cell Counter) may be used, having a tube with aperture of 140 µm diameter and counting volume of 0,3 ml.

Caution.- When the counter is installed, make sure that any electromagnetic interference is excluded. Both the screen and the time of counting shall be continuously checked.

Calibrate the apparatus before use. The relation between the volume of the particles to be counted and the threshold level above which the counts are made is thus determined. Calibrate in accordance with the manufacturer's instructions by using a standard particle suspension. Check the calibration by differential counts in some samples with counts between 300 000 and 1 000 000 cells/ml. Evidence shall be produced that the modal diameter of the cells is between 5.45 and 6.25 µm. Assess a threshold value for routine estimation, corresponding to an equivalent diameter between 4,7 and 5,0 µm, depending on the size distribution found. Check each manometer to determine whether the counts in 0,1 ml are 1/5 of the count in 0,5 ml (see Appendix 2).

6.2 Water bath, with circulation, capable of being maintained at 37 ± 1°C.

6.3 Water bath, with circulation, capable of being maintained at 80 ± 1°C.

6.4 Incubator, capable of being maintained at 30 ± 1°C.

6.5 Pipetting device, for preparation of the 1 : 100 dilution (optional, see note to 9.1).

6.6 Filter , resistant to the solvents used, with a pore size of 0,5 µm or less.

6.7 Glass or plastic tubes, for example of 100 mm length, of 16 mm diameter, round-bottomed, with straight brim and appropriate seal.

* *Coulter Electronics Ltd, Northwell Drive, Luton, Beds LU3 3RH,*

Note.- When plastic tubes are used, tests shall be made to ensure that no loss of somatic cells occurs due to adherence on the surface of the tubes. After the tubes have been rinsed, this must be repeated with filtered distilled water.

6.8 Pipetting device for dispensing 0,2 ml of fixative liquid.

6.9 Analytical balance.

7 SAMPLING

7.1 See IDF Standard 50B: 1985.

7.2 When automatic samplers are used, these shall have been properly tested.

7.3 If samples are to be stored prior to testing or preservation, this shall be done at storage temperatures of 2-6°C.

7.4 Raw milk samples may be preserved by the addition of boric acid: the final concentration in the sample shall not exceed 0,6 g/100 ml (such samples may be stored for up to a further 24 h at 6-15°C).

7.5 Immediately after sampling, samples may be fixed with formalin (see 8). This may be done using sample tubes which already contain the correct amount of the fixative liquid. The tubes shall be kept sealed to prevent evaporation of formalin.

8 PREPARATION OF TEST SAMPLE

After thorough mixing of the laboratory samples, fix the somatic cells by taking 10 ml portions of milk and mixing with 0,2 ml of the fixative liquid (5.2) dispensed by the pipetting device (6.8).

Note.- Normally fixation of the cells is done by adding formaldehyde to milk in the proportion of about 1 : 1500, that is, by the addition of 0,2 ml of fixative liquid to 10 ml of milk. Higher formaldehyde concentrations may be used (for example in the proportion of about 1: 500 by raising the amount of formaldehyde solution 35% (V/V) in the fixative liquid to 30,0 ml) but special precautions should then be taken to avoid falsely elevated counts (for example, by heating milk samples to 55°C for 50 min).

Keep the test samples for 15-18 h at 30°C in the incubator (6.4), or for 22-26 h at 18-25°C.

Note.- Fixed samples should not be stored for more than 48h at 6-15°C, in order to ensure that the precision of counts remains within the limits specified (see Appendix 1).

9 PROCEDURE

9.1 Test portion

Warm refrigerated fixed test samples in the water bath (6.2) to 20-35°C. After thorough mixing, transfer 0,1 ml portions of the test sample to tubes (6.7) and dilute with the emulsifier electrolyte mixture (5.1) to give 10 ml.

Note.- This dilution may be done either manually or by using the pipetting device (6.5). Variation should not exceed ± 1,5%. Dilution accuracy should be checked regularly by weighing, carrying out at least 20 separate determinations and using well-mixed milk.

9.2 Dispersion of fat particles

Heat the test portions (9.1) in the water bath (6.3) to 80°C for 10 min.

Check the temperature in a control blank to ensure that the test portions reach and maintain the correct temperature. Take care that the water bath contains sufficient water to keep the level of the liquid in the tubes below the water level.

Remove the test portions from the water bath and cool to 15-25°C.

Note.- Slight opalizing in the test portions after heat treatment is due to hardening of the casein micelles by formalin. These micelles have a diameter of less than 1 µm and do not affect counting.

9.3 Determination

Ensure that cell counting is carried out within 1 h of cooling the test portions (9.2). Thoroughly mix the test portions immediately before counting so as to obtain an as homogeneous a distribution of the cells as possible. Transfer the test portions to a measuring vessel, taking care that no air bubbles are produced and that sedimented cells are not retained in the tubes.

Then counting by the electronic particle counter (6.1) is performed. During counting the outer electrode of the aperture tube shall be below the surface of the liquid.

Note.- During counting the pulse monitor should be checked in order to detect possible interference. In addition the time for counting each test portion should be within tolerance.

10 EXPRESSION OF RESULTS

With a measuring volume of 0,1 ml and a dilution of 0,1 ml of milk in 10 ml of emulsifier electrolyte mixture, the number of somatic cells is given , as direct reading, in thousands per millilitre of milk.

11 PRECISION

See Appendix 1.

12 TEST REPORT

The test report shall show the method used and the result obtained. It shall also mention any operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the results.

The report shall include all details required for the complete identification of the sample.

METHOD C – FLUORO-OPTO-ELECTRONIC METHOD *(Fossomatic)*

1 SCOPE

This International Standard specifies a method for counting somatic cells in both raw and chemically preserved milk, using the Fossomatic counting instrument.

Counting of cells in unpreserved samples within the first 24 h after milking could give unreliable results with older instruments (for example Fossomatic 90 and 215).

2 REFERENCE

IDF 50B: 1985 - Milk and milk products - Methods of sampling.

3 DEFINITION

For the purpose of this International Standard, the following definition applies.

Somatic cells: Those cells that have a minimum intensity of fluorescence due to the staining of DNA in their nuclei.

4 PRINCIPLE

Mixing of the milk to be examined with buffer and stain solution. Transference of the mixture in the form of a thin film to a rotating disc, serving as an object plane for microscope. Each stained cell observed by the microscope produces an electrical pulse that is amplified and recorded.

Direct reading of the number of somatic cells in thousand per millilitre.

5 REAGENTS

All reagents shall be of recognized analytical quality. Water used shall be distilled or deionized water or water of equivalent purity.

5.1 Basic solutions

5.1.1 Dye-buffer basic solution

Dissolve 2,5 g ethidium bromide in 1 litre of deionized water in a 5 litre container. Stir gently until the ethidium bromide is completely dissolved. The process can be speeded up by heating to 40-60°C. Add 400 g of tripotassium citrate and 14,5 g of citric acid to the ethidium bromide solution. Add 4 litres of deionized water. Stir gently until the solids are completely dissolved. Add 50 ml of Triton X-100 concentrate while stirring. Keep light-proof, air-tight and cool for no longer than 90 days.

5.1.2 Polyethylene glycol mono [p-(1, 1, 3, 3-tetramethylbutyl)-phenyl]-ether

Dissolve 10 ml of polyethylene glycol mono [p-(1, 1, 3 3,-tetramethylbutyl)-phenyl]-ether (for example Triton X-100) in 1 litre of deionized water, heated to approximately 60°C. This solution can be stored air-tight and cool for a maximum of 25 days.

5.2 Working solution

5.2.1 Dye-butter working solution

Mix 1 (one) part of dye-buffer basic solution (5.1.1) with 9 (nine) parts of deionized water (enough for approximately 2700 samples).

5.2.2 Rinsing liquid

Add 10 ml of 1% (V/V) polyethylene glycol mono [p-(1, 1, 3, 3-tetramethylbutyl)-phenyl]-ether (for example Triton X-100) (5.1.2) and 25 ml of a 25% (V/V) ammonia solution to 10 litres of deionized water).

Notes.-

1 Working solutions should not be used if more than 7 days old.

2 The composition of reagents might vary depending on the counting system used. Follow exactly the instructions of the manufacturer.

6 APPARATUS

Usual laboratory equipment, and in particular:

6.1 Counting instrument, operating according to the fluorescence optical principle (for example Fossomatic*) and calibrate according to the instructions of the manufacturer. For calibration it is necessary to use milk samples the cell count of which has been made by the microscopic reference method (Method A).

Note.- Cell count standards are available.

6.2 Water bath, with circulation, capable of being maintained at 40 ± 1°C.

6.3 Sample tubes with leak-proof seal.

7 SAMPLING

7.1 See IDF Standard 50B: 1985.

7.2 When automatic samplers are used, they shall have been properly tested.

7.3 If samples are to be stored prior to testing or preservation, this shall be done at storage temperatures of 2-6°C.

7.4 If preservation is necessary, this shall be done as soon as possible after sampling, but in any case within 24 h, by addition of one of the following preservatives.

- Boric acid: final concentration in the sample not to exceed 0,6 g/100 ml (such samples may be stored for up to a further 24 h at 6-12°C).
- Potassium dichromate: final concentration in the sample not to exceed 0,2 g/100 ml (such samples may be stored for up to a further 72 h at 6-12°C). Local conditions regarding the discharge of effluents shall be observed for samples preserved with potassium dichromate.
- Sodium azide: final concentration in the sample not to exceed 0,024 g/100 ml and under the following conditions: cooling of sample immediately after sampling, and counting within 48 h of sampling.
- Bronopol: final concentration in the sample not to exceed 0,05 g/100 ml and under the following conditions: cooling of sample immediately after sampling, and counting within 72 h of sampling.

Notes.-

1 Samples already preserved with boric acid may be further preserved for up to 48 h using potassium dichromate.

2 Bulk milk shall not be examined, if the preservation has been done more than 2 days after sampling.

8 PREPARATION OF TEST SAMPLE

8.1 Store unpreserved samples for at least 24 h after milking, at a temperature of 2-6°C.

If examination of unpreserved samples has nonetheless to be performed within 24 h after milking, the samples shall be pretreated by the addition of potassium dichromate (see 7.4) and leaving them for at least 3 h.

8.2 Heat both unpreserved and preserved samples in a water bath (6.2) to 40 ± 3°C and keep them at room temperature until tested.

* *Foss Electric, Hillerhed, Denmark.*

9 PROCEDURE

9.1 Test portion

Further dilution of the test sample and preparation of the test portion take place automatically in the instrument (6.1).

9.2 Ensure that cell counting is carried out within 30 min of the end of heating (8.2). Ensure that the instrument stirrer is operating correctly so as to obtain an as homogeneous distribution of the cells as possible. If no instrument stirrer is available, thoroughly mix the test portions immediately before counting.

10 EXPRESSION OF RESULTS

The number of somatic cell is expressed in thousands per millilitre of milk

11 PRECISION

An IDF Intercomparison trial (No. 21, October 1992, 37 participating laboratories) gave the following results for r (repeatability) and R (reproducibility) (in 1000/ml):

Sample	Mean	Sr rel.	r	SR rel.	R
2	210	13,7	38,9	36,7	103,7
4	438	21,2	59,9	51,3	145,0
6	609	32,6	92,3	89,4	253,0

For the interpretation it has to be taken into account that under practical conditions the geometric mean of several (for example 3) determinations is used.

The targets for precision are in Appendix 1 (3.6).

12 TEST REPORT

The test report shall show the method used and the result obtained. It shall also mention any operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the results.

The report shall include all details required for the complete identification of the sample.

APPENDIX 1
QUALITY CONTROL IN THE LABORATORY (Instrumental methods)

1 PURPOSE

The purpose of quality control procedures is to ensure close agreement between cell counts determined in the routine way and the "true" cell count of the samples. Poor agreement may be due to random errors in individual determinations, such as may arise from inadequate mixing or inaccurate pipetting; or it may be due to systematic errors, or bias, such as that introduced by incorrect calibration of instruments. The magnitude of both kinds of error may vary with the true cell count of the sample. Figure 1 illustrates the effect of both random and systematic errors on the relationship between true and observed cell counts.

Repeatability is a measure of the variation between replicate determinations in one laboratory using the same sample. Reproducibility is a measure of the variation between determinations carried out in different laboratories using the same sample. Neither repeatability nor reproducibility, as defined by ISO 5725*, attempts to measure the bias in measurements relative to "true" values. The procedures recommended in this section aim to do both with a combination of routine checks within laboratories, and periodic collaborative trials to assess the relative performance of different laboratories.

2 ROUTINE MONITORING WITHIN LABORATORIES

2.1 Repeatability

For routine monitoring of the repeatability of counts any sample with about 500 000 cells/ml should be counted at regular intervals (for example after every 20th or 50th sample) throughout the working day. At the end of the day the coefficient of variation of the counts should be calculated. If it is greater than 5% the laboratory procedure should be checked, in particular to see that sufficient care is being taken over mixing and pipetting

2.2 Bias

In order to assess the counting bias within a laboratory, standard samples with known "true" counts must be available. Milk samples whose cell count has been estimated by microscopic counting could be used, but normal milk samples will keep for only a few days and it would be expensive to get accurate counts for fresh samples so frequently. Alternatively, standard leucocyte suspensions or milk samples suitably preserved to guarantee a shelf life of at least 1 month should be used.

Two standards with about 300 000 cells/ml and 600 000 cells/ml should be prepared and the "true" count of each sample should be determined microscopically or by electronic analysis in at least three different laboratories. The standards should be counted 5 times by each laboratory at the beginning of each series of analyses and if the mean count for either standard differs from its "true" count by more than ± 5-10% the calibration of the instrument or any other possible cause of systematic errors should be checked.

2.3 Additional requirements

In addition to 2.1 and 2.2 the following procedures should be carried out:

- calibration of the instrument with relation to the slope
- visual inspection of the instruments
- check on zero-setting
- determination of the carry-over factor.

3 COLLABORATIVE TRIALS

3.1 Objective

The purpose of collaborative trials is to obtain estimates of the repeatability of counts for the same samples of milk in different laboratories and to measure the bias in each laboratory's counts relative to the best available estimate of the "true" count of each sample. In addition to providing absolute measures of the reliability of individual counts the results of these trials demonstrate to inexperienced laboratories the levels of repeatability and bias attained in experienced laboratories.

3.2 Design

3.2.1 Ten batches of milk with cell counts spread evenly over the range 200 000 cells/ml to 800 000 cells/ml should be prepared by the organizing laboratory.

3.2.2 Four 15 ml samples of each milk should be distributed to each participating laboratory, coded in such a way that only the trial coordinators know the identity of the 40 samples.

3.2.3 Each laboratory should count each sample four times and report the individual counts to the trial coordinators.

* *International Standard ISO 5725: 1986 - Precision of test methods - Determination of repeatability and reproducibility for a standard test method by inter-laboratory test.*

3.3 Statistical analyses

In an optional description of collaborative trials the linear values of cell counts are used. Statistical analyses can also be performed using their logarithms or their square root values. The following notations are used: df for degrees of freedom; MS for mean squares; Sr for standard deviation of replicate counts; Ss for standard deviation of samples of the same milk. Bias defines the difference between the observed mean and the reference mean.

3.3.1 Calculate laboratory means and a grand mean for each of the 10 milks.

3.3.2 For each of the 10 milks in each laboratory carry out the following analysis of variance:

Source of variation	df	MS
Samples of the same milk	3	$S^2r + 4S^2s$
Replicate counts	12	S^2r

From the observed mean squares calculate the repeatability= $2,83 \times (S^2r + 4S^2s)^{1/2}$

Rank the laboratories according to the maximum r for any sample. Identify the laboratories, not exceeding 15% of the total number, with the largest maximum r. The arbitrary exclusion rate of 15% ensures that reference means in small trials are based on at least five laboratories after exclusion of repeatability and bias.

3.3.3 For each laboratory calculate the regression of its sample means on the sample grand means. From the regression line calculate the maximum bias for each laboratory within the range of observed data.

Rank the laboratories according to the maximum bias. Identify the laboratories, not exceeding 15% of the total number, with the largest maximum biases.

3.3.4 Calculate reference means for each milk excluding those laboratories identified in steps (ii) and (iii) as having the poorest repeatabilities and the largest biases.

3.3.5 For each laboratory, calculate a new regression of laboratory sample means on reference means.

3.4 Presentation

3.4.1 The laboratory means for each milk should be tabulated, and the grand means and reference means for each milk should appear at the foot of the table.

3.4.2 A single standard deviation for repeatability, pooled over all samples, should be given for each laboratory, and the laboratories should be ranked on this parameter.

3.4.3 The intercept and slope of the regression of each laboratory's means on the reference means should be given. The maximum bias within the range of observed means should also be given, and the laboratories should be ranked on this parameter.

3.4.4 Each laboratory should receive a graph on which its own individual counts are plotted against the reference means, and the 45° line and the laboratory's regression line should be shown.

3.4.5 The calculation of the reproducibility (R) or of sR is desirable.

3.5 Comparisons between trials

The distributions of repeatability and bias should be monitored from one trial to the next. For the pooled repeatabilities and bias in each trial a histogram should be constructed and the position of laboratory in the distribution. The laboratories with national responsibilities should be identified so that their absolute performance over successive trials and their performance relative to other laboratories within a trial can be seen.

3.6 Targets for precision

Analysis of international and national intercomparison trials suggests that the following figures are reasonable targets:

3.6.1 Cell count level between 400 000 and 500 000/ml:
Repeatability (r) = 2,83 x 20 000 = 57 000/ml. This is equivalent to a coefficient of variation of 4-5%.
Reproducibility (R) = 2,83 x 50 000 = 142 000/ml. This is equivalent to a coefficient of variation of 10-12%.

3.6.2 Cell count level between 100 000 and 200 000/ml:
Repeatability (r) = 2,83 x 10 000 = 28 000/ml. This is equivalent to a coefficient of variation of 5-10%.
Reproducibility (R) = 2,83 x 20 000 = 57 000/ml. This is equivalent to a coefficient of variation of 10-20%.

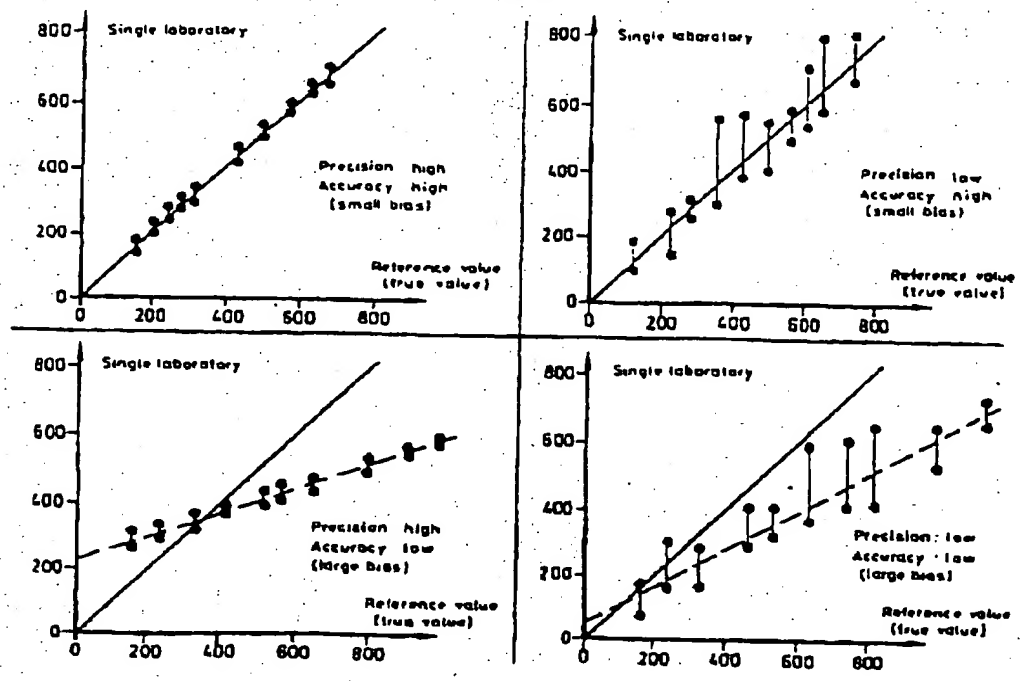

Figure 1: A graph representing the terms "precision" and "accuracy of the mean". A comparison of the counts obtained in a single laboratory with reference values, the mean of several laboratories. Ten counts each of 2 sub-samples.

APPENDIX 2
THE USE OF CELL COUNT STANDARDS

Instruments for counting somatic cells should provide not only a good repeatability (r) and reproducibility (R) in interlaboratory (collaborative) trials, but also guarantee a high accuracy of the mean or a «bias» as low as possible. As the bias is defined as the difference between the «true» value and the mean value of a number of determinations, that «true» value must be found using a method as «direct» as possible. For the determination of accuracy, suspensions of particles of defined size cannot be used. As the counting procedure in the different instruments includes steps for dilution, mixing with reagents, staining, heating, etc. in order to prepare the sample for the final counting process, the samples with the true values must have practically the same composition as the milk samples to be counted.

In recent years different approaches to prepare such samples («standards») have been made:

1. Addition of plastic particles or leucocytes to milk samples with very low numbers of somatic cells.
2. Treating milk samples with a given number of somatic cells («genuine» milk samples) in such a way that the samples can be stored for at least a few months.

The different approaches have been successful to varying degrees. Today it can be stated that the approach of adding particles to milk samples was not the method of choice. The addition of isolated leucocytes (PMN, thymocytes etc.) can be used taking into consideration that the isolation of these cells often leads to the selection of certain cell types. Thus the samples do not contain cells with the same range or distribution as in natural milk.

The specific treatment of milk samples with low, medium, and high cell content has been developed during the last years in such a way that standards ("reference samples") could be prepared which have a shelf life even under ambient temperature of several months. They can be shipped worldwide, as the treatment (combination of heat and chemical preservation) guarantees that no pathogenic microorganisms, including viruses, are present in these samples (absolute sterility).

In preparing standards on milk basis a procedure has to be described for the enumeration of the «true» number of cells in milk samples to calibrate and check the accuracy of electronic counting procedures (for example the fluoro-opto-electronic method). For this purpose the microscopic method is suitable.

Moreover it must be proven that the reference values are not biased by the treatment (example see Figure 2). For storage (time, temperature) follow the instructions of the manufacturer.

Using this procedure standards can be made available with a guaranteed true cell count. The laboratories have the advantage that microscopically-counted milk samples are [illegible] readily and can be used whenever it seems to be necessary without doing the time-consuming and difficult work of microscopic counting of cells in the given laboratory.

For including cell standards (milk standards) in the quality [illegible] laboratory, the following recommendations can be given:

1 Determination of repeatability (r)

Repeated determination of the cell counts in a milk sample (preserved or not preserved) with a cell content between 400 000 or 500 000/ml.

2 Determination of reproducibility (R)

Participation in interlaboratory trials.

3 Determination of the accuracy («bias»)

Use of standards with true values, determined by microscopic counts. The standards can be used a few times daily to determine whether the counted value is within certain limits of the value stated for the standard (for example, ± 5-10% with a cell count level of 400 000-500 000/ml).

There will not always be a close relationship between too high and too low counts of the standards and respective deviations in the milk samples counted. It has to be taken into consideration that the standard samples are well preserved and of «good quality». Deviating numbers (too high or too low) of somatic cells in the routine samples might be influenced by too high numbers of bacteria, too long periods of preservation using certain chemical preservatives (for example, sodium azide) and other factors. Achieving the numbers of somatic cells given on the milk standards can only ensure that the whole counting system including sample preparation is working correctly. The cell counting laboratory has to take care that the «quality» of the routine samples counted is such that an exact calibrated instrument can count the «accurate» cell numbers in these samples.

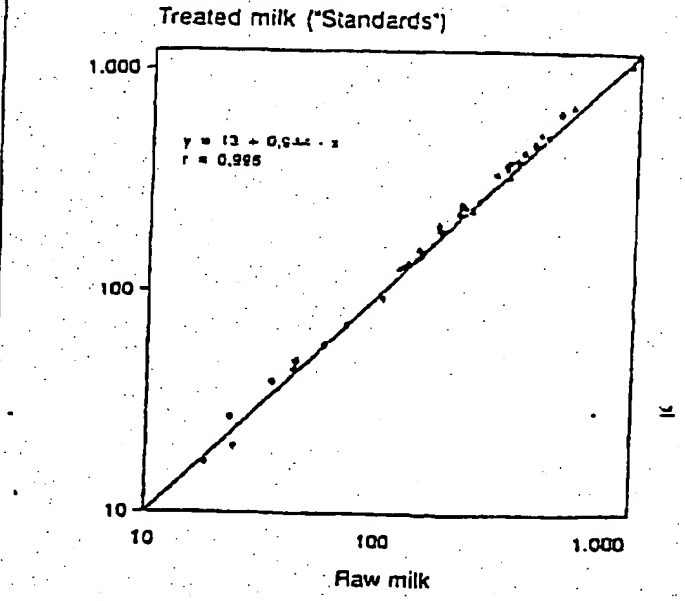

Figure 2: Counting of somatic cells in milk: comparison of preserved raw milk samples and thermo-chemically treated identical milk samples ("standards")